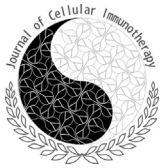
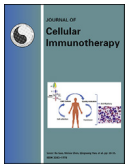


Available online at www.sciencedirect.com

ScienceDirect

Journal of Cellular Immunotherapy 2 (2016) 52–57



http://www.elsevier.com/locate/jocit

Phagocytic activity of neutrophil is induced by granulocyte colony stimulating factor and interleukin-15 in leukemic animal model

Bhaskar Bhattacharjee, Ashish Kumar Singha, Debasish Maiti*

Immunology Microbiology lab, Department of Human Physiology, Tripura University, Suryamaninagar, 799102, Agartala, Tripura, India

Received 28 September 2015; revised 9 November 2015; accepted 14 January 2016
Available online 23 January 2016

Abstract

In leukemia, secondary infection is common and after chemotherapy the number of normal active neutrophil reduced significantly. Therefore, to avoid side effects of chemotherapy and to induce neutrophil activity and number, in this study, we have treated with G-CSF plus IL-15 in combination to N−N′ Ethylnitrosourea (ENU) induced leukemia in BALB/c mice. After 5 months of ENU treatment in 3−4 weeks old mice at 80 mg/kg body weight, leukemia was confirmed by histology of blood smear co-treatment started with cytokines for 5 days. The *in vitro* phagocytosis activity of neutrophil from spleen was assayed using Dalton's lymphoma ascites as target cell. Using real time polymerase chain reaction (RT-PCR), Toll like receptor 4 (TLR4) and toll like receptor 9 (TLR9) expression of neutrophil was also measured. Our data suggests that G-CSF plus IL-15 induce the phagocytic activity of neutrophil. TLR4 and TLR9 expression was induced in neutrophil after treatment with cytokines together which was significantly reduced in leukemia. In conclusion, G-CSF plus IL-15 has potential role as therapeutic immunomodulators for neutrophil activity in leukemia.

Keywords: Leukemia; Granulocyte colony stimulating factor (G-CSF); Interleukin-15 (IL-15); N−N′-Ethyl-Nitrosourea (ENU); Toll like receptor 4 (TLR4); Toll like receptor 9 (TLR9)

1. Introduction

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells. These immature cells accumulate in blood and organs but all of them are not able to carry out the normal functions of blood [1]. Due to increase the abnormal blood cells in the peripheral blood the normal function of normal immune cells are altered.

Specific doses of ENU, also known as N-ethyl-N-nitrosourea (chemical formula $C_3H_7N_3O_2$), can induce mixed type of leukemia (both the myelogenous and lymphogenous) [2]. Neutrophils, the most abundant immune cell in blood quickly arrive at sites of infection and form the first line of defense following infection. The key role of neutrophils is the antimicrobial effector functions and the ability to produce cytokines to initiate inflammatory responses and chemokines to induce trafficking of immune cells [3].

During the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, environmental exposure [4], and some cancers [5], neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation. In leukemia, the immune system is in depressed condition and the infection by different microorganism in that condition, is prevented generally by the neutrophils. In acute leukemic condition the number of neutrophil decreased significantly [6]. Neutrophils migrate through the blood vessels, then through interstitial tissue, following chemical signals such as Interleukin-8 (IL-8) and C5a by the process called chemotaxis [7]. The neutrophils in chronic myeloid leukemia (CML) exhibit defects in several functions [8].

* Corresponding author.
E-mail address: debasish.maiti@tripurauniv.in (D. Maiti).
Peer review under responsibility of Shanghai Hengrun Biomedical Technology Research Institute.

http://dx.doi.org/10.1016/j.jocit.2016.01.002

The innate immune system has evolved to discriminate between self and foreign pathogens through a process that relies, to a great extent, on an evolutionary conserved family of pattern recognition receptors, including TLRs. At least 13 TLRs have been identified in mammals to date and their ligands are predominantly pathogen-associated molecular patterns, a limited set of conserved molecular patterns that are unique to microbes [9]. TLRs are type 1 transmembrane receptors that play important role in innate immune recognition of pathogens [10]. Recognition of conserved molecular patterns found on microbes by these invariant, germ line encoded receptors leads to a signal transduction cascade that results in cellular activation and cytokine release in both immune and non-immune cells.

Granulocyte colony stimulating factor (G-CSF) is a lineage specific hematopoietic growth factor that initiates the differentiation and proliferation of committed progenitor cells into mature neutrophils. G-CSF exerts its proliferative effect mainly at the stage of myeloblast-promyelocyte, and also stimulates the release of mature bone marrow neutrophils from storage pools into the peripheral circulation [11]. In vivo studies of G-CSF administration reveals enhanced adhesion on nylon wool, phagocytosis, luminal enhanced chemiluminescence, degranulation and expression of cell surface antigens [12]. One study also showed promotion of neutrophil survival *in vitro* [13]. After allogeneic stem cell transplantation the patient with invasive fungal disease also improved with G-CSF treatment [14].

IL-15 can induce phagocytosis, cytoskeleton rearrangement, gene expression, *de novo* protein synthesis and can delay apoptosis in human neutrophils [15]. Production of chemokines, cytokines and natural inhibitors is increased in IL-15 induced neutrophils, including CXCL8 (IL-8) [16], IL-1beta, IL-1RII and IL-1Ra [17]. IL-15 has also been shown to induce the redistribution of ICAM-3 and p-selectin glycoprotein ligand-1 (PSGL-1) to the uropods in neutrophil [18]. The mechanisms involved in IL-15-induced activation of human neutrophils are not fully understood. However, IL-15 was shown to activate NFk-β [19], and to induce the phosphorylation of Syk and its physical association with IL15R-α [20].

As the combined treatment approach with IL-15 and G-CSF to leukemia animal has not been well studied to evaluate their immunological response, our focus of this study was to evaluate the neutrophil immunological activity such as phagocytic activity during pre and post treatment with combinations of cytokines in ENU induced leukemia model. The molecular changes like the expression of pattern recognition molecules like TLR4, TLR9 and alteration of PTK activity in both the conditions were evaluated.

2. Materials and methods

2.1. Materials

Nitroblue tetrazolium (NBT) Hydrocloric acid (HCL), and Pyridine were purchased from SRL chemicals, India. Lipopolysaccharide (LPS), and N-ethyl-N′-nitrosourea (ENU) were purchased from Sigma Aldrich, USA. Leishman's stain was purchased from Loba chemicals, India.

Dalton's lymphoma ascites are the kind gift from Dr. Nabyendu Murmu, CNCI, Kolkata India. RPMI-1640, Fetal Bovine Serum was purchased from Hi-media, India. Recombinant mouse interleukin-15 (rmIL-15), recombinant mouse granulocyte colony stimulating factor (rmG-CSF) were purchased from ImmunoTools, Germany. Percol was purchased from GE Health Care Life Sciences, Uplasa, Sweeden. PureZOL™ RNA isolation reagent, iTaq™ universal SYBR® Green Supermix, kit, iScript™ c-DNA synthesis kit were purchased from BioRad, USA.

2.2. Animals

Healthy BALB/c male mice were obtained from National Centre for Laboratory Animal Sciences, (NCLAS), NIN, Hyderabad, India. Animals were divided into four groups having six animals in each group. Groups were divided as Mock control, ENU challenged, and control along with combination cytokine treatment and ENU along with combination cytokine treatment. These mice were kept and maintained specific pathogen free condition in Tripura University Animal House as per guidelines of Institutional Animal Ethical Committee. Food, dietary supplements and water were provided *ad libitum*.

2.3. Induction of leukemia

We have induced leukemia previously in our lab following the reference of Law et al., 2003 and detailed protocol has been published [21]. In short leukemia was induced by injecting N-ethyl-N′-nitrosourea (ENU) intraperitoneally (i.p.) to 7−10 days old mice, at the dose of 80 mg/kg body weight concentration at two times in one week interval. The peripheral blood and bone marrow slides were prepared for both control and treated group and stained with Leishman's stain. Total count and differential count of leukocytes were also done in all the four groups to confirm leukemia [21].

2.4. Cytokine treatment

ENU treated groups were followed up for leukemia induction after 4 months in one week interval. After 5 months of ENU injection leukemia was confirmed by peripheral blood smear observation. The two cytokines, rmG-CSF at the dose rate of 10 μg/kg body weight [22] and rmIL-15 at the dose of 5 μg/kg body weight [23] were injected (i.p.) in animals of one control and one ENU treated group after confirmation of leukemic induction for consecutive five days.

2.5. Isolation of splenic neutrophils

Neutrophils were isolated from the spleen as previously described [21]. In short, the splenic suspension was made by smashing the spleen gently by two frosted glass slides in a

sterile petri dish containing 37 °C RPMI-1640 media with 10% FBS after collecting the spleens from sacrificed mice. Two layered Percol™ gradient was used to isolate neutrophils [24]. Cell suspension was layered on 1.11 gm/ml density and 1.089 gm/ml density percol and centrifuged at 900 g for 30 min at room temperature. The interphase between two layers consisting neutrophils were washed in phosphate buffer saline (PBS) and finally suspended in complete RPMI-1640 media. Total number of cells were counted by haemocytometer.

2.6. Phagocytic activity assay for neutrophils

Dalton's lymphoma ascites were used as target cells. Neutrophils were sized at 3−4 × 10^6/ml. Daltons lymphoma cells are collected and sized into 3−4 × 10^5/ml respectively and mixed effector: target = 10:1 (4 × 10^6: 4 × 10^5) in the presence of 1% NBT in normal saline (200 µl)/sample and 15 µl of LPS (Stock: 10 mg/ml) and incubated at 37 °C for overnight (18 h) at 5% CO2 air environment [25]. The reaction was stopped by adding by 0.1 N HCl (chilled) (2 ml). The cells were pelleted by centrifugation and supernatant discarded. The pellet was resuspended by adding 3 ml of pyridine in each tube and boiled for 15 min in boiling water bath. Tubes were cooled and centrifuged and the supernatant taken to read at 530 nm wavelength. Experiment was repeated thrice and results expressed as mean ± SD.

Percent phagocytosis was calculated as:

$$\%\,\text{phagocytosis} = \frac{\text{Optical density (O.D.) of test}}{\text{O.D. of control} - \text{O.D. spontaneous lysis}} \times 100$$

2.7. Real time polymerase chain reaction (RT-PCR)

Total RNA was isolated from the neutrophils using PureZOL™ RNA isolation reagent, according to the manufacturer's protocol. In brief, isolated neutrophils of each group 2.5−4.5 × 10^7 cells were used for isolation in triplicate. Cells were pelleted down by centrifuging at 3000−5000× g for 5 min and supernatant containing media was discarded. 1 ml PureZOL reagent was added in each tube and incubated at room temperature for 5 min 0.2 ml of chloroform for each 1 ml of PureZOL was added in each tube and incubated for 5 min at room temperature. The tubes were centrifuged at 12,000× g for 15 min at 4 °C. The whole mixture was separated into three phases, an upper colorless aqueous phase, a white interphase and a lower organic phase. The upper aqueous phase was collected carefully in a new 1.5 ml sterile tube and 0.5 ml of isopropyl alcohol was added in each tube, mixed well and incubated for 5 min at room temperature. Mixtures were centrifuged at 12,000× g for 10 min at 4 °C. The RNA was precipitated as white pellet on the bottom of the tube and the supernatant was discarded. The RNA pellet was washed with 1 ml of 75% ethanol twice and air dried. The RNA was reconstituted in nuclease free DEPC-treated water and quantitated using synergy H1-hibrid plate reader.

The complementary DNA was synthesized from isolated RNA was done using iScript™ c-DNA synthesis kit, according to the manufacturer's protocol. 1 µg of RNA was used in c-DNA synthesis for each group in triplicate. In brief, 20 µl of reaction mixture components were, 4 µl of 5× iScript™ reaction mix, 1 µl of iScript™ reverse transcriptase, 1 µl of RNA solution and 14 µl of nuclease free water. Reaction protocol: 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C. After completion of the samples were stored at −80 °C for future downstream RT-PCR experiments.

Real time polymerase chain reaction (RT-PCR) was done to evaluate the expression of Toll like receptor 4 (TLR4, forward 5′-CTGGCTGGTTTACACGTCCA-3′: reverse 5′-GCTGCA GCTCTTCTAGACCC-3′) and toll like receptor 9 (TLR9, Forward 5′-GCCTGAGCCACACCAACATCCT-3′ reverse 5′-CCAGACCTTGGAACCAGGAAGA-3′) gene along with endogenous control glyceraldehyde 3 phosphate dehydrogenase (GAPDH, forward 5′ CACCACCCTGTTGCTGTAGCC-3′: reverse 5-ACCACAGTCCATGCCATCAC-3′) using iTaq™ universal SYBR® Green Supermix, kit, according to the manufacturer's protocol in Step One Plus 96 well real time PCR system, Applied Biosystems, USA. Total reaction mixture of 10 µl of was prepared using 5 µl of iTaq™ universal SYBR® Green Supermix (2×), 1 µl forward and reverse primer (10 µM concentration each) of gene of interest, 1 µl of c-DNA template (1 µg) and 2 µl of nuclease free water. A negative control was run along with each set of primers to eliminate unwanted exogenous amplification like primer dimer and contamination. To discriminate specific amplification from nonspecific amplification, melting curve analysis was performed at the end of each PCR assay. To determine the starting c-DNA amount, purified PCR products with known concentrations were serially diluted. PCR conditions used for all the primer sets were as follows: 95 °C hot start for 5 min, followed by 40 amplification cycles of 95 °C for 20 s (denaturing), 59°C−55 °C (annealing temp, according to the Tm values of the set of primers), 72 °C for 30 s (extension). A melt curve analysis was also done using instruments default settings to detect any exogenous contamination with negative controls for each set primers. GAPDH was used as endogenous control. Expression of gene of interest was calculated using the Ct values of the corresponding genes in reference with the endogenous control, GAPDH. All the experiments were done 5 times. The RT-PCR products with equal volume were further run through agarose gel for all the three genes to observe the band pattern with a 100 bp ladder. Experiment was repeated 5 times and results expressed as mean ± SD.

3. Statistical analysis

All the readings were taken 3−5 times repeat of the same experiment and expressed as mean ± SD using MS excel 2010 software. Data were analysed by student's t-test using GraphPad Prism online software.

4. Results

4.1. Neutrophil activity is reduced in ENU induced leukemia

Secondary infection is common in leukemia patient. The active neutrophil may help to protect this infection and in this study we studied the activity of neutrophil in ENU induced leukemic BALB/c mice. The neutrophils were isolated from spleen of ENU induced leukemic mice [23] and used to assay the *in vitro* phagocytic activity against Dalton's lymphoma ascites cells as target. We have shown earlier that the number of neutrophil decreased in leukemia [20] and in this study, the phagocytic activity of the neutrophil was evaluated. This activity was significantly reduced in leukemia which is reinstated upon co-treatment with IL-15 plus G-CSF (Fig. 1).

4.2. Neutrophil activity was regulated through TLR4 and TLR9 expression in leukemia

During tumour development, immune suppression is one of the major cause of secondary infection and growth of tumour cells. The phagocytic activity of neutrophils depends on expression of TLR4 and TLR9 [26]. More the expression of these proteins in neutrophil better will be the recognition of pathogen or tumor cell. Both pro-tumor and anti-tumor effect of neutrophil has been reported [27].

The expression of TLR4 (Fig. 2A and B) and TLR9 (Fig. 3A and B) was significantly reduced in ENU induced leukemic group of animals in compared to the control group. But co-treatment with IL-15 and G-CSF, both TLR4 and TLR9 expression increased significantly which also increased the phagocytic activity of neutrophil.

5. Discussion

ENU is being used as a potent carcinogenic agent [28,29] which can induce several cancer including brain tumour [30], reproductive tumour [31], leukemia etc. in animal model. In this present study, BALB/c mice were used to induce leukemia by injecting ENU [32,33]. ENU, a carcinogen, can cause immune suppression in animal [34,35] and so the tumor load increased gradually in animal. To combat against tumor cells the manipulation is required to increase the number of normal immune cells in peripheral blood. G-CSF is a growth factor to proliferate and differentiate granulocyte cells. It is also reported that G-CSF can induce the expression of TLR9 to recognize pathogen [36]. IL-15 has also important role in innate immune response.

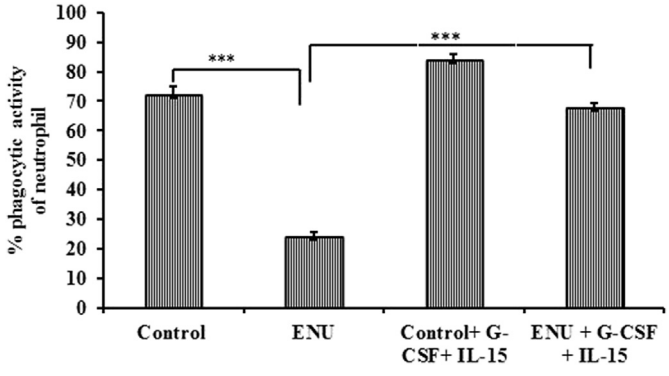


Fig. 1. **Phagocytic assay**. Bar diagram showing % phagocytic activity of neutrophil in different groups of animal. In leukemia, the functional activity was reduced around 3.5 times compared to control. (p= < 0.0001 between control and ENU, p= < 0.0001 between ENU and ENU + G-CSF + IL-15) This activity was regained in leukemic animal after co-treatment with G-CSF and IL-15 in combination. (p < 0.05 indicates *, p < 0.01 indicates **, p < 0.001indicates ***).

One of the major immunological responses of neutrophils is the phagocytic activity. Neutrophil has tumoricidal activity besides the phagocytosis to pathogen [26]. TGF-β inhibition causes the tumoricidal activity of the neutrophil [37]. By increasing the production of TNF-α, MIP1α, H_2O_2 and NO, neutrophil can kill the tumor cells in absence of TGF-β. Through tumor antibody dependent cell mediated cytotoxicity neutrophil can kill tumor cells though there are lacks of evidences for this activity.

Our results showed that the phagocytic activity of neutrophils was down regulated in ENU induced leukemic mice probably due to inhibitory character of carcinogen and/or the lack of recognition of the tumor cells. This recognition is dependent on the expression of TLRs. In this study both TLR4 and TLR9 expression was significantly reduced in leukemic mice. In this study, *in vitro* phagocytosis activity was significantly reduced in the leukemia group. Also reduced number of neutrophils in ENU group interferes with the functional activity. But after the co-treatment with G-CSF and IL-15 in combination, phagocytic activity restored even more than control levels. Inducing tumor cells to release G-CSF, enhance the neutrophil population towards the tumor site and can regress the tumor load *in vivo* [38]. Interleukin-15 plays a very crucial role in phagocytic activity of neutrophils by recruiting dendritic cells and generating cytotoxic T lymphocytes against cancer cells [39]. Along with that, the ability of G-CSF to consistently induce white blood cell counts confirms its role as a potent hematopoietic growth factor, particularly for cells of the granulocytic lineage [40]. Recent studies have suggested that human and mouse neutrophils used to kill target cells using TLRs, specially TLR4 and TLR9 as a mechanism of suppression and used in recent therapeutic approach [41].

In this study, the expression of both TLR4 and TLR9 has reduced in leukemic condition, and after co-treatment with G-CSF and IL-15, the expression level was almost equal to the control group. Altogether, co-treatment with G-CSF and IL-15 increases the phagocytic activity of neutrophil in leukemic animal, may be through their TLR4 and TLR9 expression pathway.

6. Conclusion

In tumor, immune suppression is common and neutrophil can participate in tumor killing. Treatment with G-CSF and IL-15 in combination induced the phagocytic activity of

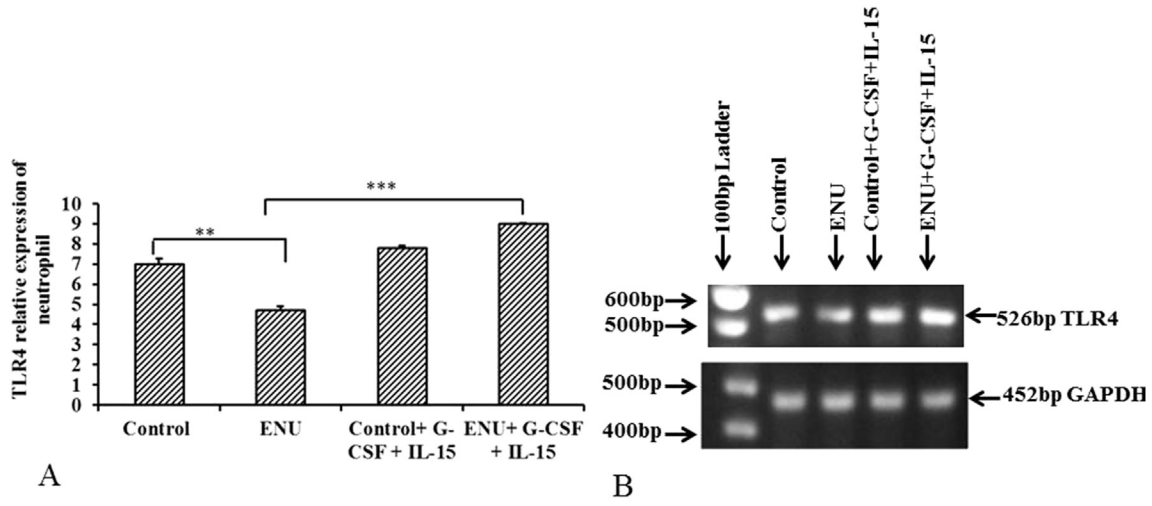


Fig. 2. **Expression of TLR4 in neutrophil. A**. Bar diagram showing relative mRNA expression of TLR4 in neutrophils isolated from spleen of different groups of animals. ($p = 0.0085$ between control and ENU, $p = 0.0005$ between ENU and ENU + G-CSF + IL-15) **B**. RT-PCR products were loaded taking same volume in agarose gel. ($p < 0.05$ indicates *, $p < 0.01$ indicates **, $p < 0.001$indicates ***).

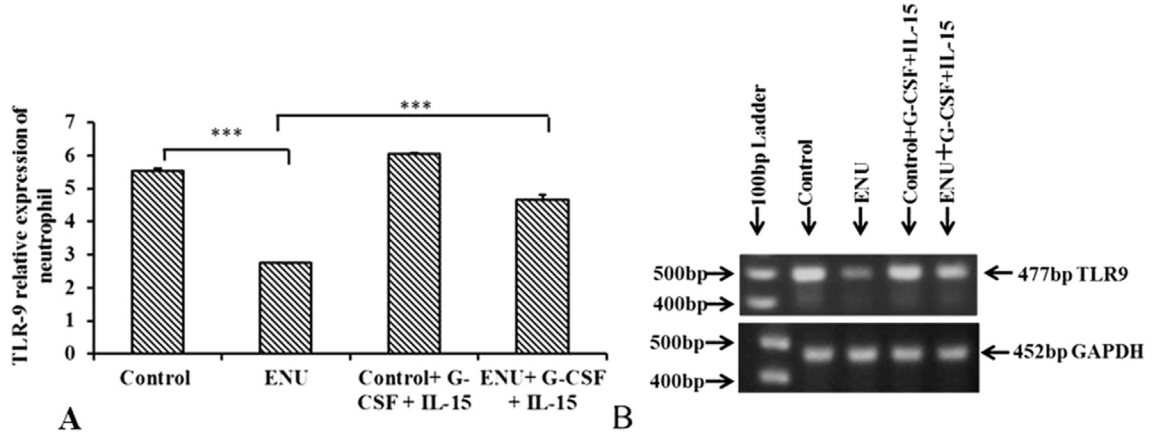


Fig. 3. **Expression of TLR9 in neutrophil. A**. Bar diagram showing relative mRNA expression of TLR9 in neutrophils isolated from spleen of different groups of animals. $p = 0.0002$ between control and ENU, $p = 0.0002$ between ENU and ENU + G-CSF + IL-15 **B**. RT-PCR products were loaded taking equal volume in agarose gel. ($p < 0.05$ indicates *, $p < 0.01$ indicates **, $p < 0.001$indicates ***).

neutrophil in leukemic animal. This treatment also increases the TLR4 and TLR9 expression on neutrophil in BALB/c leukemia model.

Conflict of interest

Authors declared that there is no conflict of interest.

Authors' contributions

BB performed the experiment, analyse the data and wrote the manuscript. AKS performed the experiment. DM conceived the plan of work, designed and performed the experiment, and wrote the manuscript.

Acknowledgements

Project is funded by DBT as State biotech Hub; Grant No. BT/04/NE/2009 Dt 22-10-2010, Govt. of India. We are grateful to Dr. Nabyendu Murmu, CNCI, Kolkata for providing the Dalton's Lymphoma cell line, Sanjoy Nath, M.D. Department of Pathology, Tripura Medical College and BRAM Hospital, for the conformation of onset of leukemia.

References

[1] Pierson BA, Miller JS. The role of autologous natural killer cells in chronic myelogenous leukemia. Leuk Lymph 1997;27(5–6):387–99.
[2] Druckrey H, Ivanković S, Preussmann R. Teratogenic and carcinogenic effects in the offspring after single injection of ethylnitrosourea to pregnant rats. Nature 1966;210:1378–9.
[3] Yamashiro S, Kamohara H, Wang JM, Yang D, Gong WH, Yoshimura T. Phenotypic and functional change of cytokine-activated neutrophils: inflammatory neutrophils are heterogeneous and enhance adaptive immune responses. J Leukoc Biol 2001;69:698–704.
[4] Jacobs L, Nawrot TS, de Geus B, Meeusen R, Degraeuwe B, Bernard A. Subclinical responses in healthy cyclists briefly exposed to traffic-related air pollution: an intervention study. Environ Health 2010;9:64.
[5] Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res 2008;14(21):6735–41.

[6] Silver RT, Beal GA, Schneiderman MA, Mccullough NB. The role of the mature neutrophil in bacterial infections in acute leukemia. Blood 1957;12(9):814−21.
[7] De Larco JE, Wuertz BR, Furcht LT. The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. Clin Cancer Res 2004;10(15):4895−900.
[8] Balasubramanian N, Advani SH, Zingde SM. Protein kinase C isoforms in normal and leukemic neutrophils: altered levels in leukemic neutrophils and changes during myeloid maturation in chronic myeloid leukemia. Leuk Res 2002;26(1):67−81.
[9] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783−801.
[10] Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002;14:103−10.
[11] Hollingshead LM, Goa KL. Recombinant granulocyte colony stimulating factor(rG-CSF): a review of its pharmacological properties and prospective role in neutropenic conditions. Drugs 1991;42:300−30.
[12] Hariss AC, Todd WM, Hakney MH, Ben-Ezra J. Bone marrow changes associated with recombinant granulocyte- macrophage and granulocyte colony stimulating factors: discrimination of granulocytic regeneration. Arch pathol lab Med 1994;118:624−9.
[13] Rapoport AP, Abboud CN, DiPersio JF. Granulocyte- macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF); receptor biology, signal transduction and neutrophil activation. Blood Rev 1992;6:43−7.
[14] Wan L, Zhang Y, Lai Y, Jiang M, Song Y, Zhou J, et al. Effect of granulocyte-macrophage colony-stimulating factor on prevention and treatment of invasive fungal disease in recipients of allogeneic stem-cell transplantation: a prospective multicenter randomized phase IV Trial. J Clin Oncol 2015;33(34):3999−4006.
[15] Adachi S, Kubota M, Lin YW, Okuda A, Matsubara K, Wakazono Y, et al. In vitro administration of granulocyte colony stimulating factor promotes neutrophil survival in vitro. Eur J Haematol 1994;53:129−34.
[16] Girard D, Paquet ME, Paquin R, Beaulieu AD. Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils: modulation of phagocytosis, cytoskeleton rearrangement, gene expression and apoptosis by IL-15. Blood 1996;88:3176−84.
[17] Musso T, Calosso L, Zucca M, Millesimo M, Puliti M, Bulfone-Paus S, et al. Interleukin-15 activates proinflammatory and antimicrobial functions in polymorphonuclear cells. Infect Immun 1998;66:2640−7.
[18] Jablonska E, Piotrowski L, Kiluk M, Jablonski J, Grabowska Z, Markiewicz W. Effect of IL-15 on the secretion of IL-1beta, IL-1Ra and sIL-1RII by neutrophils from cancer patients. Cytokine 2001;16:173−7.
[19] Alonso-Lebrero JL, Serrador JM, Domínguez-Jiménez C, Barreiro O, Luque A, del Pozo MA, et al. Polarization and interaction of adhesion molecules P-selectin glycoprotein ligand 1 and intercellular adhesion molecule 3 with moesin and ezrin in myeloid cells. Blood 2000;95:2413−9.
[20] Ratthé C, Girard D. Interleukin-15 enhances human neutrophil phagocytosis by a Syk-dependent mechanism: importance of the IL-15R alpha chain. J Leukoc Biol 2004;76:162−8.
[21] Bhattacharjee B, Singha AK, Maiti D. Role of G-CSF plus IL-15 on neutrophil population in peripheral blood modulating protein tyrosine kinase activity in leukemic mouse. Int J Curr Res 2015;04(7):15180−6.
[22] Berger C, Berger M, Hackman RC, Gough M, Elliott C, Jensen MC, et al. Safety and immunologic effects of IL-15 administration in nonhuman primates. Blood 2009;114(12):2417−26. Sep 17.
[23] Gascon P, Fuhr U, Sörgel F, Kinzig-Schippers M, Makhson A, Balser S, et al. Development of a new G-CSF product based on biosimilarity assessment. Ann Oncol 2010;21:1419−29.
[24] Boxio R, Bossenmeyer-Pourié C, Steinckwich N, Dournon C, Nüsse O. Mouse bone marrow contains large numbers of functionally competent neutrophils. J Leukoc Biol 2004;75:604−11.
[25] Law S, Maiti D, Palit A, Chaudhuri S. Role of biomodulators and involvement of protein tyrosine kinase on stem cell migrate on in normal and leukaemic mice. Immunol Lett 2003;86(3):287−90.
[26] Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood 2003;102:2660−9.
[27] Alyssa DG, Houghton AM. Tumor-Associated neutrophils: new targets for cancer therapy. Cancer Res 2011;71(7):2411−6.
[28] Justice MJ, Noveroske JK, Weber S, Zheng B, Bradley A. Mouse ENU mutagenesis. Hum Mol Genet 1999;8(10):1955−63.
[29] Dexter TM, Schofield R, Lajtha LG, Moore M. Studies on the mechanisms of chemical leukaemogenesis. Br J Cancer 1974;30(4):325−31.
[30] Briançon-Marjollet A, Balenci L, Fernandez M, Estève F, Honnorat J, Farion R, et al. NG2-expressing glial precursor cells are a new potential oligodendroglioma cell initiating population in N-ethyl- N-nitrosourea-induced gliomagenesis. Carcinogenesis 2010;31:1718−25.
[31] Stoica G. ENU-induced ovarian cancer. Methods Mol biology™ 2002;188:305−14.
[32] Chatterjee S, Basak P, Das M, Das P, Pereira JA, Dutta RK, et al. Kinetic impairment of haemopoietic stem cells in experimentally induced leukemia and aplastic anemia: an inverse correlation. J stem cells 2009;4:179−89.
[33] Singha AK, Bhattacharjee B, Maiti D. Cytotoxic activity of T lymphocytes is induced upon stimulation with IL-3 plus GM-CSF in animal leukemia model. Leuk Res 2015. S0145-2126(15):30364-7.
[34] Chen D, Li Z, Chen T. Increased expression of miR-34a in mouse spleen one day after exposure to N-ethyl-N-nitrosourea. J Appl Toxicol 2011;31:496−8.
[35] Vaquero J, Zurita M, Oya S. Immune-cell system provides the development of extraneural tumors in the ethyl-nitrosourea model of neurocarcinogenesis. Cancer Lett 1998;123:95−8.
[36] O'Mahony DS, Pham U, Iyer R, Hawn TR, Liles WC. Differential constitutive and cytokine-modulated expression of human toll-like receptors in primary neutrophils, monocytes, and macrophages. Int J Med Sci 2008;5(1):1−8.
[37] Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell 2009;16:183−94.
[38] Colombo MP, Lombardi L, Stoppacciaro A, Melani C, Parenza M, Bottazzi B, et al. Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition in vivo. Neutrophils discriminate between G-CSF producing and G-CSF nonproducing tumor cells. J Immunol 1992;149:113−9.
[39] Kandasamy M, Bay BH, Lee YK, Mahendran R. Lactobacilli secreting a tumor antigen and IL15 activates neutrophils and dendritic cells and generates cytotoxic T lymphocytes against cancer cells. Cell Immunol 2011;271(1):89−96.
[40] Demetri GD, Griffin JD. Granulocyte colony-stimulating factor and its receptor. Blood 1991;78:2791−808.
[41] Stier S, Maletzki C, Klier U, Linnebacher U. Combinations of TLR ligands: a promising approach in cancer immunotherapy. Clin Dev Immunol 2013:14. http://dx.doi.org/10.1155/2013/271246. Article ID 271246.